Support for the amendments to claims 15, 16, 18 and 20-22 can be found, for instance, at page 1, line 21, and page 6, first paragraph. These claims have been amended to more clearly point out that the vectors of the present invention are particularly suited for use in myeloma cells, and may comprise more than one heterologous gene.

New claims 23-30 have been added to more clearly define the claimed plasmids. Support for new claims 23-30 can be found on pages 8-11 in the section entitled Vectors. Claim 31 has been added to point out a preferred selection method of the present invention. Support for new claim 31 can be found on page 13, lines 29-35. New claims 32-39 correspond to original claims 15-22, and have been added to preserve the originally claimed subject matter in a more appropriate form for examination.

Claims 15-39 are now active in the application and are believed to be in allowable condition.

I. Relying on 35 U.S.C. § 101, claims 15-22 stand rejected under the judicially created doctrine of obvious-type double patenting as being unpatentable over claims 21 and 22 of U.S. Patent No. 5,122,464. Applicants respectfully request reconsideration.

The Examiner states that although the conflicting claims are not identical, they are not patentably distinct because

D

the subject matter of the claims overlap, and that both the patent and the application claims contain the vector having the GS gene operably linked to a weak promoter upstream from a heterologous gene operably linked to a strong promoter. This generalization regarding the patent claims is simply not true. Claims 21 and 22 of the patent are directed to plasmids that include either the GS minigene or SV40-GS transcription unit from pSVLGS.1. These claims state nothing about a weak promoter nor a heterologous gene nor the organization of the plasmid. Furthermore, since the instant claims are directed to plasmids specifically designed to confer glutamine-independent growth to myeloma cells, and the instant specification discloses that the vector claimed in the patent, pSVLGS.1, does not confer glutamine-independent growth to myeloma cells (page 13, lines 7-11), the patent claims cannot render the instant claims obvious. Withdrawal of this rejection is respectfully requested. In addition, since myeloma cells are also lymphoid cells, this rejection should also not be applied to any of the newly added claims.

II. Relying on 35 U.S.C. § 102(b), original claim 15 remains rejected as being anticipated by Wilson et al. (WO 87/04462). Applicants respectfully request reconsideration of this rejection.

The Examiner has upheld the rejection of claim 15 set forth in Paper No. 7, page 2, where it was stated:

> Wilson further discloses that the vectors can be used to endow a cell line with the ability to survive in a medium lacking glutamine by transforming a host cell completely lacking or reduced in GS activity with the vector containing the GS gene and that the procedure is particularly, but not exclusively, applicable to myeloma cells (page 11, fourth paragraph).

Claim 15, as newly amended, recites a vector that confers glutamine independence to a transformed myeloma cell line, comprising a GS gene and a gene or genes encoding a protein(s) heterologous to said myeloma cell line, wherein the genes are arranged such that said GS gene can be expressed and glutamine independent myeloma colonies can be produced.

Although Wilson et al. contemplate the use of the vectors disclosed therein for use in myeloma cells (page 11, paragraph 4), such a use is never described. The vectors of Wilson et al. were only used to transfect CHO cells. As one of skill in the art would know, CHO cells are not of lymphoid origin. In fact, applicants have since found that the vectors disclosed in Wilson et al. do not render myeloma cells glutamine independent (see page 13, Example 1, first paragraph).

Specifically, vectors pSVLGS1 and pSV2GS were previously described in WO 87/04462 (see instant specification, page 13,

first full paragraph). Vector pSVLGS1 contains a GS minigene under the control of a SV40 late region promoter, and pSV2GS contains a GS cDNA sequence under the control of an SV40 early region promoter. As described in the instant specification beginning on page 13, although the pSVLGS1 vector was successfully used in CHO cells in the previous disclosure, this vector could not be introduced into myeloma cells to confer glutamine independent growth. This may be a result of promoter strength, since plasmids comprising stronger promoters gave increased transfection frequencies (page 13, lines 19-23). Furthermore, although plasmid pSV2GS gives an intermediate transfection efficiency in myeloma cells without any heterologous genes present (page 13, lines 21-23) the presence of heterologous genes on the same vector substantially reduces the frequency with which transfected colonies can be isolated (page 16, lines 19-24). Only applicants' disclosure regarding the organization of the plasmid rectifies these deficiencies.

Thus, the disclosure in Wilson et al. is not sufficient for the skilled artisan to confer glutamine independence to myeloma cells, which are lymphoid cells. In fact, the contemplation in Wilson et al. that the vectors could be used in myeloma cells is in direct conflict with what has been subsequently found. The disclosure of Wilson et al. was therefore prophetic at the time, which is supported by the

fact that transfection of myeloma cells was never described. The Federal Circuit clarified such issues in **Ashland Oil, Inc. v. Delta Resins & Refractories, Inc.**, 776 F.2d 281, 227 USPQ 657 (Fed. Cir. 1985), where it was decided:

> (1) A reference must be considered for all it teaches, including disclosures that teach away from the invention as well as disclosures that point toward the invention.
>
> (2) The test whether a compound described in the prior art may be relied upon to show obviousness is whether the prior art provided an enabling disclosure with respect to the disclosed compound. Because the evidence showed that a certain compound was a "hypothetical structure", it was not persuasive of obviousness.

Although the above citation refers to obviousness, the same standard, if not higher, should apply to anticipation. Wilson et al. definitely errs in the supposition that plasmid pSVLGS1, which contains the GS gene expressed from a weak promoter, confers glutamine independence to myeloma cells. Thus, Wilson et al. "teaches away" from applicants' claimed invention. Furthermore, although Wilson et al. hypothetically disclose vectors that will confer glutamine independence to myeloma cells, the instant specification provides evidence that the vectors of the prior art are insufficient in this regard. Only the instant specification describes the modifications and considerations required to achieve the end result. For these reasons, reconsideration and withdrawal of this rejection is respectfully requested.

III. Relying on 35 U.S.C. §103, claims 16-22 stand rejected as being unpatentable over Wilson as applied to claim 15 above and further in view of Ringold (USPN 4,656,134) and Foecking. Applicants respectfully request reconsideration of this rejection.

Applicants' vectors according to the claims are submitted to be neither disclosed in nor suggested by any reasonable combination of the teachings of the applied references for the reasons which follow.

The Examiner maintains this rejection on page 3 of the Office Action where she states:

> Wilson discloses on page 34 that the efficiency of expression is affected by the order in that the tPA gene was more efficiently expressed when in the same orientation in the vector as is the GS gene than when the two genes were in opposite orientations.

First of all, if the Examiner will kindly refer to page 34 of Wilson et al., she will see that this comparison was made using vector pSVLGS1, which, as established in the discussion set forth above, does not confer glutamine independence to myeloma cells even in the absence of a heterologous gene. A teaching that transcriptional order and direction may affect gene expression in an expression system that does not work to begin with is inconsequential. Such a secondary teaching in the absence of a primary teaching that discloses a vector <u>as currently claimed</u> does not make up for the deficiency.

The Examiner goes on to state that:

> Ringold discloses in column 3, lines 35-48, that the promoter for the amplifiable gene should be weaker than the promoters for the succeeding genes, and that, in this manner, greater amplification may be achieved when applying the selective pressure with greater expression of succeeding genes. Thus Ringold achieves the same end as applicants, which is increased amplification of the target gene.

The Examiner is requested to review the language of the instant claims, which, as it applies to this rejection and line of reasoning, has not been amended. The instant claims are directed to plasmids that confer glutamine independence to myeloma cells. The objective of the claimed invention is first and fore-most to confer glutamine-independence to myeloma cells. The statement that "Ringold achieves the same end as applicants" demeans the primary accomplishment of applicants' invention, which is to provide vectors that confer glutamine independence to myeloma cells. Ringold does not disclose vectors that confer glutamine independence to myeloma cells. The amplification of the heterologous gene is a secondary benefit of the claimed vectors which is expressed in the specification but not in the claims.

To know from the prior art that considerations of promoter strength may be important does not render the claimed vectors obvious. In fact, contrary to what is taught in Ringold, as stated above, applicants' vectors may comprise a GS gene and a heterologous gene, each transcribed from the

same type of promoter, as is now recited in new claim 27 and shown for myeloma cell line CMGS.cLc-13/7 in the specification at Table 7. The vector pCMGS.cLc is described at page 10, lines 14-17, and comprises the GS gene and an immunoglobulin light chain gene, both under the control of a hCMV-MIE promoter.

What is primarily important in the claimed vectors is that the strength of GS gene expression is strong enough to confer glutamine independence to myeloma cells. The promoter of the heterologous gene and the direction of transcription, although important, are not chosen with amplification considerations in mind, but with regard to stability in myeloma cells, since transcription read-through and promoter occlusion can effect the level of GS gene expression.

The fact that Ringold could not possibly suggest or render obvious the claimed invention is even more clear when you consider the promoters used in Ringold. While some of applicants' claimed vectors contain the GS gene operably linked to a weak promoter such as the SV40 early region promoter (which is only "weak" in relation to the hCMV-MIE promoter), Ringold uses the SV40 early region promoter as the second, strong promoter for the heterologous gene and the MMTV promoter as the first, weaker promoter for the amplifiable gene (see Figure 1, column 7, lines 5-8). This combination, although relatively weaker-to-stronger as are

some of applicants' promoter combinations, would not have conferred the level of GS expression required for selection of myeloma cells, let alone amplification of the GS gene.

Support for the fact that MMTV is known to be a much weaker promoter than is the SV40 early region promoter can be found in the attached U.S. Patent by Sobczak et al. at page 10, last paragraph (Exhibit A), where it is stated that the MMTV LTR is a weak promoter and the early promoter of SV40 is a strong promoter. In fact, Sobczak et al. use the plasmid and dhfr amplification protocol disclosed by Ringold to amplify an HBV gene. However, the weaker MMTV promoter would not be effective for GS gene expression in myeloma cells, since, as argued previously above, the SV40 early promoter gave only an intermediate efficiency with regard to recovery of transfectants, while the use of a weaker promoter (i.e. the SV40 late region promoter) was not effective (page 13, lines 7-11).

The Examiner goes on to argue that:

> [A]lthough neither Wilson nor Ringold specifically address the problem of "transcriptional interference", Ringold in particular noted the importance of promoter strength and gene order and therefore inherently both recognized the problem and determined the solution.

Again, this statement does not take into consideration the fact that the promoters used in Ringold et al. would not have been effective to confer glutamine independence to myeloma

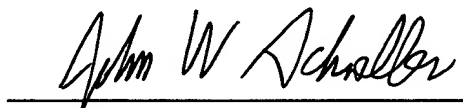
cells, and that it is not merely the relative strength of the two promoters that is important, but also the base strength of the promoter operably linked to the GS gene. Thus, Ringold has not "recognized the problem" with regard to myeloma cells, and certainly has not "determined the solution".

In conclusion, neither Ringold nor Wilson et al. disclose vectors comprising the GS gene and a heterologous gene that confer glutamine independence to transfected myeloma cells. Furthermore, Ringold certainly does not make up for the deficiencies of Wilson et al., and the combined references do not render obvious the claimed invention. For these reasons, reconsideration and withdrawal of the rejection under 35 U.S.C. 103 over Wilson in view of Ringold is respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, it is requested that the rejections of record be reconsidered and withdrawn, and that the Application be found to be in allowable condition. The Examiner is requested to telephone the undersigned Counsel should any of the pending claims be found to be allowable, or if she believes that a conference would be of value in expediting the prosecution of the Application.

Respectfully submitted,



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<=2> GET 1st DRAWING SHEET OF 6

Jun. 28, 1994

Composition useful for the fabrication of vaccines

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APPL-NO: 662,993

FILED: Feb. 28, 1991

FOR-PRIOR:
Mar. 7, 1984 France 84 03564

REL-US-DATA:
Continuation of Ser. No. 431,718, Nov. 3, 1989 now abandoned Which is a continuation of Ser. No. 163,185, Feb. 25, 1988 now abandoned Which is a continuation of Ser. No. 800,650, Jan. 7, 1987 now abandoned

INT-CL: [5] A61K 39#12; A61K 39#00; A61K 39#42

US-CL: 424#227.1;

CL: 424;

SEARCH-FLD: 424#89, 239, 85.8, 86; 530#325, 350

REF-CITED:

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PRIM-EXMR: Waddell, Frederick E.

ASST-EXMR: Weddington, K.

LEGAL-REP: Finnegan, Henderson, Farabow, Garrett & Dunner

ABST:
The invention concerns a composition useful for the manufacture of vaccines containing particles having the immunogenic properties characteristic of the antigen HBsAg, these particles being more particularly characterized by the fact that the said particles equally contain a receptor for polymerized human albumin. They are obtained by transformation of human or animal cells by a vector containing a DNA sequence coding for the S and pre-S regions of a genome of viral hepatitis B, this DNA sequence being placed under the direct control of a promoter permitting the effective transcription of the said sequence in the human or animal cells transformable by the said vector.

NO-OF-CLAIMS: 12

EXMPL-CLAIM: <=10> 1

NO-OF-FIGURES: 7

NO-DRWNG-PP: 6

PARCASE:

This application is a continuation of application Ser. No. 431,718, filed Nov. 3, 1989; now abandoned which is a continuation of application Ser. No. 163,185, filed Feb. 25, 1988, now abandoned, which is a continuation application under 37 C.F.R. 1.62 of prior application Ser. No. 800,650, filed as PCT/FR85/00044, Mar. 7, 1985, now abandoned entitled COMPOSITION USEFUL FOR THE FABRICATION OF VACCINES CONTAINING PARTICLES BEARING THE SURFACE ANTIGEN OF THE HEPATITIS B VIRUS AND THE RECEPTOR OF POLYMERIZED HUMAN SERUM ALBUMIN, ANIMAL CELLS CAPABLE OF PRODUCING SUCH PARTICLES AND A PROCESS FOR OBTAINING THEM, National Phase of PCT/FR85/00044, filed Mar. 7, 1985.

SUM:

The invention concerns a composition useful for the fabrication of vaccines, containing or formed by approximately spherical polypeptide particles, at least as concerns most of them, these particles having immunogenic and immunologic properties characteristic of the surface antigen of the virus of viral hepatitis B. This antigen is often designated by the abbreviation HBsAg or yet more simply HBs. It equally concerns the eucaryotic cell lines, preferably animal, capable of excreting into their culture medium polypeptide particles of the kind indicated above with elevated production yields and the means, notably modified vectors, permitting such cell lines to be obtained.

One will recall first that the serum of chronic carriers of the virus of hepatitis B (HBV) contains empty viral envelopes in the form of particles or filaments of 22 nm diameter and sometimes the complete infectious virions, spherical particles of 42 nm. The viral envelope bears the surface antigen (HBsAg) and the presence of infectious virions is generally accompanied by a soluble antigen called antigen (HBeAg).

The polypeptide composition of the viral envelope has been much studied (Robinson W. S. (1977) Ann. Rev. Microbiol. 31, 357-377). It includes a major polypeptide present in glycosylated form (GP29) and non-glycosylated (P25) and at least three minor polypeptides called GP33, GP36 and P41. The polypeptides GP33 and GP36 may have the same sequence in amino acids. GP36 would possess only one supplementary sugar residue (Stibbe W. and Gerlich W. H. (1983) J. Virology 46, 626-628). The relative quantity of minor proteins in relation to the major polypeptide varies from one plasma to another. It is very superior in HBeAg positive serums rich in viral particles. The polypeptides GP33 and GP36 may then represent several % of the proteins of the envelope while they represent less than 1% in non-infectious HBeAg negative serums (Stibbe W. and Gerlich W. H. (1982) Virology 123, 436-442).

The major polypeptide of the envelope is constituted of 226 amino acids and is coded by the gene S. The polypeptide sequence of GP33 and GP36 may be coded by the S gene and a part of the pre-S region. In this hypothesis, this polypeptide sequence would have the same C-terminal end as the major polypeptide and would contain a supplementary sequence of 55 amino acids in the N-terminal position (Stibbe W. and Gerlich W. H. (1983), J. Virology 46, 626-628).

Recently, it has been shown that the viral particles isolated from an HbeAg positive serum contain a receptor for polymerized human albumin (pHSA) (Machida A. et al (1983) Gastroenterology 85, 268-274). This receptor may be carried by polypeptides GP33 and GP36. Thanks to this receptor, the pHSA may form a bridge between the viral particle and the hepatocyte, thus permitting the attachment of the virus and it's penetration into the hepatic cell. The appearance of the anti-receptor antibodies may be essential in the process of "clearance" of the virus. In fact, the sero-conversion HBeAg/anti-HBe, initial step of recovery, is accompanied by the appearance of these antibodies. while these are absent during the evolution towards chronicity (Pontisso P. et al (1983) J. of Virolgical Methods 6, 151-159).

In parallel, one observes the disappearance of the antigens corresponding to the pHSA receptor, which appears to bear evidence that the expression of the corresponding gene does not normally occur except in specific situations

(notably during the step of the replication of the virus). It is this which is corroborated again by the analysis of the empty natural envelopes of the hepatitis B virus, such as those contained in vaccine preparations currently commercialized and obtained from blood serum from donors who, in the past, have been exposed to the hepatitis B virus. In fact, the analysis by gel electrophoresis on polyacrylamide in the presence of sodium dodecylsulfate (SDS) of polypeptides obtained after dissociation of the natural particles at 100 degrees C. for 5 minutes, in the presence of dithiothreitol (DTT), does not reveal the presence of polypeptides having elevated molecular weights, notably of the order of 34,000. In the same way, the presence of such high molecular weight polypeptides has not been observed in the compositions of particles possessing the immunologic and immunogenic properties of the HBs antigen, such as obtained by the transformation of animal cells transformed by genetic engineering techniques by means of vectors which have been described for example in the European patent application No. 38 765.

The invention has for a goal to furnish vaccine compositions having reinforced protection properties with regard to the hepatitis B virus. It also has for a goal to furnish cell lines transformed by the techniques of genetic engineering, which may be maintained in culture and which are apt to excrete the active principles of these reinforced vaccine compositions into their culture medium, and this with yields of production much higher than those permitted by most of the genetically transformed cultures currently available. And lastly, it has for a goal to furnish the means (vectors and process) permitting the obtaining of such cell lines from eucaryotic cell lines, notably from mammals, of the sort which are capable of being maintained in culture.

DRWDESC:
BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1 and 2 present the essential successive steps of the production of a plasmid conforming to the invention, particularly appropriate to the effective transformation of animal cells, such as the CHO line;

FIG. 3 is a schematic representation of the amplification of DNA sequences coding for the polypeptide immunogen in CHO cells according to the invention;

FIGS. 4 and 5 show the results of the gene amplification of the cells previously transformed under the conditions of the process according to the invention;

FIG. 6 contains a curve representing the variation of the quantity of HBsAg antigen produced as a function of the levels of specific HBV-RNAs observable in the producing cells;

FIG. 7 reports in schematic fashion the successive harvests of the cells in stationary phase.

DETDESC:

The invention results from the observation that it was possible to induce the expression of genetic sequences which, in the genome of the virus of hepatitis B code for the pHSA receptor, under conditions however very far from those which are prevalent under the specific biological situations which were evoked

above. Last, it has been noted that the association in the same vaccine composition of the antigen HBs with the polypeptide fragments carrying an antigenic determinant appearing to correspond to this receptor, led to the reinforced vaccine compositions in question above.

The composition useful for the manufacture of vaccines according to the invention which contains approximately spherical polypeptide particles (or is formed by these particles), at least as concerns most of them (if not all), which have immunogenic and immunologic properties characteristic of the antigen HBsAg, which have sizes of 18 to 25 nm, notably from 20 to 22 nm, and densities permitting their isolation in a density zone of 1.20-1.22 g/ml in CsCl based density gradient, and a level of total purity for there to an absence of any Dane's particles and HBe antigens, including the HBc, and more particularly characterized by the fact that the said spherical particles equally contain a receptor for polymerized human albumin. More particularly, the polypeptide particles of the invention may be seen to contain substantial proportions of polypeptides having molecular weights of the order of 34,000 daltons, these polypeptides preferably constituting a proportion superior to 10%, and preferably superior to 20% of the total quantity of polypeptide constitutive of the aforesaid particles. Preferably yet, the composition according to the invention is exempt of any component of human origin, such as one finds in human blood serums. It is a matter essentially of compositions of particles having the immunogenic properties of the antigen HBsAg and of the pHSA receptor which are excreted into their culture medium by cell lines maintained in culture and which have been previously transformed by a vector containing the appropriate coding sequences, the transformation having been accomplished under conditions permitting the effective expression of peptide sequences bearing the immunogenic sites as well as the HBs antigen and the pHSA receptor.

The invention equally concerns the vectors appropriate to the transformation of the eucaryotic cell lines, more particularly of human or animal cells in culture to render them apt to produce the above indicated immunogenic particles. These vectors, which contain a DNA sequence coding for the S and pre-S regions of the genome of the virus of viral hepatitis B, are characterized in that the said sequence of DNA is, within this vector, placed under the direct control of an exogenous promoter of known capacity to permit the effective initiation of the transcription of the genes directly under its control in the eucaryotic cells, notably human or animal, for which the said vectors are intended. One may refer for example to the article by Galibert and coll., 1979, Nature, vol. 281, p. 646-650, about the said DNA sequence. It includes notably the S gene itself preceded by the pre-S sequence containing approximately 165 triplets and whose first nucleotides appear in the partial formulation of the said sequences indicated below: [See Original Patent for Chemical Structure Diagram]

The exogenous promoter used is distinct or foreign vis-a-vis the "endogenous" promoter, normally associated with the S and pre-S genes in the genome of the hepatitis B virus. When these cells originate from the monkey, it is advantageous to have recourse to a promoter issuing from the virus SV40, whose capacity is known to permit the effective initiation of the transcription of adjacent genes in the monkey cells. Advantageously, this promoter corresponds to the "precocious" promoter of the SV40 virus, which normally controls the expression of the small T antigen and equally of the large T antigen.

The invention is not limited however to the utilization of this particular promoter, although it has given particularly favorable results with regard to the production by transformed cells of polypeptides carrying the immunogenic determinants characteristic of the HBs antigen and a pHSA receptor, and to their excretion into the culture medium used. One may equally have recourse for example to the late promoter of SV40 (which controls the expression of the proteins VP1, VP2 and VP3). One may refer to the restriction map of the SV40 virus (J. Tooze, Ed. DNA Tumor Viruses, Cold Spring Harbor, N.Y., 1980, chaps 2-5), to appreciate the relative positions of these promoters and the genes coding for the different antigens which are associated with them.

It goes without saying that one may substitute for the SV40 promoters any other type of promoter known to possess or which may be discovered (to have) the capacity to promote the transcription in the cell lines used of the said sequences coding for the aforesaid S and pre-S regions, as soon as they are placed under their control, with for result the incorporation of these sequences with this promoter in the genome of the receiving cells and/or the capacity conferred to the receiving cells thus transformed to synthesize and to excrete substantial quantities of polypeptides having the immunogenic properties of HBsAg and of the pHSA receptor, the capacity thus acquired then being transmitted to the successive generations issuing from these cells.

The said transformed cells will be called "stable" when the character acquired by the cell lines according to the invention to synthesize the said polypeptides is transmitted from one generation of cells to the others, over at least 10 generations.

As examples of other promoters susceptible to being used, one might mention for example the early promoter of the polyome or the LTR promoters of different retroviruses or again the EA promoter of the adenovirus.

As is well known, the promoters taken from the genomes of the virus from which they originate are preferably accompanied by activating "sequences" which normally precede them (relative to the direction of the transcription of the gene sequences normally placed under their control). As an example of the activating sequences, one may refer to the article in Science, 1983, vol. 219, pages 626 to 631, and Nature 1982, vol. 295, pages 568 to 572.

Advantageously, the aforesaid DNA sequence coding for the aforesaid pre-S and S regions is placed immediately behind a DNA fragment constituted of the promoter and the activating sequence permitting the normal transcription of the pre-S or S sequence. The aforesaid fragment includes notably from 300 to 400 base pairs according to the type of promoter and activating sequences utilized.

Preferably yet, the vector according to the invention also contains a sequence of DNA or a label, such as an enzyme, this label being itself under the control of a distinct promoter weaker than the first promoter mentioned above. This label is preferably constituted of a gene or sequence of DNA coding for dihydrofolate reductase (dhfr). It is to be noted that this label lends itself with a particular advantage to the amplification of the number of copies of this vector in the cells which it is capable of transforming, under the conditions which will be indicated further on.

The second promoter is in most cases equally an exogenous promoter. It may however equally be constituted of one of the natural promoters contained in

the genome of viral hepatitis B. A particularly preferred promoter is that which comes from the LTR (a sequence called "Long Terminal Repeat" described in Nature, 1981, 294, 228-232) of the mouse mammary tumor virus (MMTV).

The invention also concerns the cell lines transformed by the vectors such as they have been defined above and which are apt to excrete into their culture medium the immunogenic particles defined above. In particular, it concerns cell lines having a capacity of production of at least 1 microgram, and preferably of at least 10 micrograms of HBsAg per 10<6 > cells and per 24 hours. The invention concerns in particular the lines characterized by the specific HBV-RNAs which are of sizes greater than 2.1 kb, notably of the order of 2.2 to 2.8 kb, preferably 2.6 kb, that one may detect there when the transcription of the S and pre-S regions occurs, normally initiated in the pre-S region. The preferred lines are those in which one may equally detect specific HBV-RNAs having sizes greater than 2.1 kb, notably of the order of 2.2 to 2.8 kb, and preferably yet of the order of 2.4-2.6 kb. In general, one will simultaneously detect specific HBV-RNAs having sizes of the order of 2.1 kb, such as that which results from the transcription of the pre-S and S regions, normally initiated in the pre-S region under the control of an endogenous promoter, under the particular biological conditions referred to above (notably during the course of the replication of the virus of hepatitis B) and specific HBV-RNAs having sizes corresponding to the higher values which have been indicated.

Preferably, the lines according to the invention are formed from mammalian cells, notably from CHO cells.

The invention also concerns a process of production of such cell lines susceptible of being maintained in culture, this process includes the transformation of these lines with a vector such as defined above and the isolation of those of the cultures which simultaneously express the sequences coding for the S and pre-S regions of the genome of the virus of hepatitis B.

Of preference, one has recourse to the complete vector which includes the label under the control of a second promoter, the culture of the transformed lines being then realized in the presence of an inhibitor of the marker, the level of this inhibitor in the culture medium being regulated to a concentration sufficient to provoke an amplification of a number of copies of the gene coding for the label in certain at least of the colonies in culture, amplification which allows both the selection of resistant colonies and the obtaining of the cellular clones which secrete accrued quantities of particles presenting the immunogenic activities characteristic of HBsAg and a pHSA receptor.

Advantageously, the label is constituted of a sequence of DNA (or gene) coding for dhfr and the inhibitor is constituted of methotrexate.

The utilization of a second promoter weaker than the first favors the amplification of the number of copies of the DNA sequence coding for HBsAg and the pHSA receptor. In effect it is in general the clones which contain the greatest number of copies of the sequence coding for the label and, as a consequence, in the preferred cell lines of the invention the greatest number of sequences coding for the pre-S and S regions, which are themselves to survive in the culture medium containing the inhibitor.

The effective clones are advantageously selected in culture media containing from 0.5 to 40 micrograms of methotrexate, notably from 1 to 25 micrograms. It

is desirable to realize a pre-amplification in media (which are) poorer in inhibitor, notably from 5 to 150 nM of methotrexate, the clones selected at the end of this first step then being cultured in media containing the higher levels of methotrexate indicated above.

The complementary characteristics of the invention will appear yet in the course of the description which follows of the preferred construction of the vectors used for the transformation of the cell lines of animal origin, from the production of cell lines having a high yield of excretion of particles according to the invention and results susceptible of being obtained.

I-CONSTRUCTION OF VECTORS

A-Construction of pSVS Plasmid (FIG. 1)

It permits the expression of the S region (the pre-S region and S gene) under the control of the early promoter of the SV40 virus.

The BglII fragment of 2.3 kb has been excised from the pCP10 described in the European patent application No. 81 400634. The pCP10 plasmid is schematized in FIG. 1. The thick and hatched parts are of HBV-DNA origin and the part in thinner lines come from the pBR322 plasmid. The part indicated by the arc a corresponds to the aforesaid fragment of 2.3 kb. pCP10 contains a dimer in tandem head to tail of the HBV genome. This fragment commences ten nucleotides before the ATG of the pre-S region and it terminates 1.1 kb after the TAA stop codon of the S gene. It contains the putative site of the polyadenylation of the mRNA of the HBsAg, and the initiation site of the transcription of the S gene.

One utilizes the early promoter of SV40 contained in the plasmid pSV2 gpt (ATCC 37145) in which the gene of the gpt of E. coli is fused to a PvuII HindIII fragment of 348 bp of the early region of SV40. This fragment includes, other than the origin of the replication of SV40 (SV40 ori), the early and late promoters, the site of the initiation of the transcription of the early messengers and the 72 bp repeated in tandem. pSV2 gpt is schematized in FIG. 1. The thick black parts originate from SV40, the parts on thinner lines from pBR322. The thick white parts contain the pre-S and S regions. The hatched parts correspond to unused sequences of HBV.

The BglII fragment of 2.3 kb, issued from pCP10, has been inserted into the unique HindIII site of the plasmid pSV2 gpt by the ligature of the fragment ends of the DNA rendered open by DNA polymerase (Klenow). After the study of the recombinant plasmids a clone has been selected (pSVH4) in which the region coding for HBS was oriented relative to the SV40 promoter, in a fashion to permit it's expression. The construction has been verified by restriction maps and the nucleotide sequence at the junction of the two fragments SV40 and HBV has been determined.

The plasmid pSVS has been constructed by the ligature of three fragments issued from three plasmids:

1) the fragment of 2.5 kb, PvuI-KhoI issued from pSVH4 (arc b),

2) the fragment of 1.9 kb, XhoI BglII, issued from pCP10 (arc c),

3) the fragment of 1.0 kb, BamHI PvuI, from pBR322 (arc d).

The resulting plasmid, pSVS (5.4 kb) differs from pSVH4 by the absence of the gpt region and from the SV40 DNA sequences following after this gpt sequence, and by the presence of the EcoRI-BamHI fragment of pBR322.

B-Construction of the Plasmid pSVS dhfr (FIG. 2)

One procedes by genetic recombination of the fragments defined under a) and b) hereafter.

a) The Fragment Containing the dhfr

The plasmid pMTV dhfr described by G. Ringold and coll. (1981) J. of Molecular and Applied Genetics 1: 165-170 (filed with the C.N.C.M. the 7.03.84 under the No. I-286), after linearization by the enzyme PvuI, has then been digested partially by the enzyme HindIII, liberating several fragments of which one of 4400 bp (arc e) containing:

the LTR of MMTV,

the cDNA of dhfr,

the intron of the tAg of SV40,

the site of polyadenylation of the early genes of SV40,

an EcoRI-PvuI fragment of pBR322.

b) The HBV DNA Fragment

The pSVS plasmid has been digested by PvuI then HindIII, thus liberating a fragment of 4676 bp (arc f) containing:

the PvuI-PvuII containing the origin of the replication of pBR322;

the origin of the replication and the early promoter of the SV40 virus;

the 2.3 kb BglII fragment of HBV containing the parts coding for the pre-S and the S gene, as well as the polyadenylation signal of this gene;

the BamHI-HindIII fragment of pBR322.

c) Ligature of the Fragments

After purification, these two fragments are joined by the homologous sites PvuI and HindIII, thus re-establishing the resistance to ampicillin of pBR322.

In the recombinant vector, the units of transcription of the S region and of the dhfr gene are oriented in the same direction and separated by around 300 bp of pBR322.

The cDNA coding for the dhfr may also be obtained from the pSV2 dhfr (ATCC 337146), pSV3 dhfr (ATCC 37147) or pSV5 dhfr (ATCC 31148).

The placement of the gene of the dhfr under the control of a weak promoter (LTR of MMTV) and of the HBV gene under the control of a strong promoter (early promoter of SV40) increases the efficiency of gene amplification. The nucleotide sequence of the junction between the pre-S region and the early promoter of SV40 has been verified (FIG. 2).

II-TRANSFECTION OF ANIMAL CELLS

1) Transfer and Expression of the Plasmid pSVS dhfr in CHO dhfr< - > :

CHO dhfr< - > cells (Urlaub G. and Chasin L. A. (1980) P.N.A.S. 77, 4216-4220) (filed with C.N.C.M. the 7.03.84 and under No. I-287) have been transfected according to the technique of Graham and Van der Eb (1973, Virology 52, 456-467) modified by Parker and Stark (1979, J. Virology, 31, 360-369). The production of HBsAg by the CHO dhfr< + > has been tested by radioimmunoassay (RIA) in the supernatant of cellular cultures. 60% of the dhfr< + > clones were HBsAg< + > . The rate of HBsAg production was relatively weak between 1 and 20 ng/10<6 > cells per 24 hours.

2) Amplification by the HBV Sequences

It has been obtained in propogating the CHO HBsAg< + > clones in the presence of methotrexate (MTX), analog of folic acid and dhfr inhibitor. In effect, the resistance to MTX is due principally to an amplification of the number of copies of the dhfr gene, which leads to an augmentation of the quantity of the dhfr enzyme. The HBV sequences and the dhfr gene being carried by the same plasmid and thus integrated together in the DNA of the host cells, these are co-amplified with the dhfr sequences in the MTX resistant clones. Like for the dhfr, the augmentation of the number of the copies of the HBV sequence is accompanied by an augmentation of the synthesis of HBsAg by the cell.

The gene amplification has been effected in two steps according to the strategy described in FIG. 3. FIG. 4 represents the results of the first amplification step and FIG. 5 the results of the second step. This has been effected from the most productive clones, issued from the first step. In FIGS. 4 and 5, the dotted bars represent the rate of synthesis of HBsAg before amplification and the bars in solid lines after amplification. The concentrations of MTX are reported in these figures. They were 50, 100 or 140 nM in the first step and 1.5, 10 or 25 micro-M in the second. In total, around 150 MTX resistant clones have been screened for the production of HBsAg. As shown in FIGS. 4 and 5, the amplification of HBsAg production was highly variable from one clone to another, in the first as well as the second step.

The number of copies per cell of the HBV sequence present in different clones has been evaluated according to the hybridization technique on a cellulose filter or analogue, according to the technique called "dot blot", utilizing as a probe cloned HBV-DNA labeled with <32> P. For one same dose of MTX, this number was extremely variable from one clone to the other, going from 100 to 500.

The organization of the HBV sequences in the cellular clones has been analyzed according to the technique of Southern. The HBV sequences were present in the integrated form; the electrophoresis profiles were reasonably comparable from one clone to another. The differences observed between the selected clones at one same dose of MTX does not permit the explanation of the conflict between the number of copies of HBV-DNA and the level of synthesis of HBsAg (results not reported). A comparable analysis has been effected for the dhfr gene. For one same dose of MTX, the number of copies per cell was equally highly variable from one clone to the other (results not reported). For a given clone, there was a parallel amplification of the HBV and dhfr sequences.

The quantity of specific RNAs of HBV was equally been analyzed by molecular hybridization. Contrary to the results obtained for DNA, the quantity specific HBV RNAs was proportional to the rate of HBsAg production (FIG. 6). Moreover, for one same dose of MTX (50 nano-M), the quantity of dhfr specific RNAs was

constant in the different clones analyzed (FIG. 6). The specific HBV RNAs have been analyzed according to Northern. Two RNAs, a major one of 2.1 kb, the other minor at 2.5 kb, have been demonstrated. The study of these RNAs by S1 nuclease mapping has shown the existence of a major initiation in the region pre-S at the 3157 position corresponding to the 2.1 kb RNA, the other minor in the SV40 promoter corresponding to the 2.5 kb RNA.

3) Analysis of the HBsAg Particles

The analysis has been effected on the particles produced by the 37BA5. The supernatants corresponding to several harvests of 24 hours obtained in stationary phase have been put together. The HBsAg particles have been purified by two successive ultracentrifugations in a CsCl density gradient followed by a velocity ultracentrifugation in a sucrose gradient. The HBsAg particles have a density of 1.20. Observed under the electron microscope, they appeared like spherical particles of a mean diameter of 22 nm, morphologically similar to particles of human origin. No tubular form was observed.

After dissociation of the particles at 100 degrees for 5 minutes in the presence of DTT, the polypeptides have been analyzed by gel electrophoresis on polyacrylamide in the presence of SDS. The gel has been revealed by coloration with silver salts. Three bands have been observed, corresponding to polypeptides of 22,300, 26,100 and 34,000 daltons. The relative intensity of the coloration of these three bands has permitted the evaluation of 54%, 19% and 27% the proportions of the three proteins. After labeling in vivo by <35> S methionine, the purified particles have been immunoprecipitated by an anti-HBs serum, then the proteins were analyzed by electrophoresis. The three polypeptides described above were detected on the autoradiogram and in the same proportions.

The presence of the receptor for pHSA at the surface of the particles has been tested by radioimmunoassay in solid phase according to the technique of Hansson and Purcell (1979), Infect. Immun. 26, 125), modified by Pontisso et al (J. of Virological Methods 6, 151-159). The detection of this receptor is revealed to be positive in the supernatant as well as on the purified particles (Table 1):

TABLE 1

Supernatant of the culture (13 micrograms/ml)	2,805 cpm
Purified particles (12 micrograms/ml)	1,564 cpm
Positive control serum HBe positive	2,536 cpm
Negative control serum HBsAg negative	678 cpm

One may equally utilize the technique described by Machida et al (1983), Gastoenterology 85, 268-274.

III-IMMUNIZATION ASSAYS

After addition of aluminum hydroxide Al(OH)3 to the concentration of 0.1%, a preparation of particles has been used to immunize Balb/c mice. As shown in Table II, the immunogenic power of cellular particles is identical to that of the vaccine against hepatitis B, commercialized under the trademark HEVAC B

(and obtained from human donors from serums having levels of natural HBsAg). The 50% effective dose is of 0.04 microgram for the cellular particles and of 0.03 microgram for HEVAC B (trademark of the Institut Pasteur).

TABLE II

	Vaccine HEVAC B		Particles HBsAg CHO	
HBsAg	* Seroconversion > 50 mu RIA Number of	Anti-HBs antibodies Geometric mean	* Seroconversion > 50 mu RIA Number of	Anti-HBs antibodies Geometric mean
Injected doses	mice	mu RIA	mice	mu RIA
0,312 micrograms	20/20	> 388	20/20	> 505
0,078 micrograms	19/20	> 212	14/20	> 87
0,019 micrograms	6/20	> 24	6/20	> 24
0 controls	4/20			
	DE500.04 micrograms		DE500.03 micrograms	

n mu RIA = units defined in the AUSAB test commercialized by ABBOTT -

It results then from what has preceded that the vector pSVS dhfr permits, after integration into the cellular DNA, the synthesis and excretion of the empty envelopes of the HBV virus in the form of 22 nm particles. The elimination of a great part of the HBV genome and in particular the gene coding for the protein of the capsid excludes the production of complete infectious viral particles. This vector equally carries a unit of transcription of the gene of murine dhfr which, after introduction into dhfr< - > , permits them to be multiplied in the presence of MTX by a phenomenon of gene amplification.

The technique of amplification by resistance to MTX, utilized here, has permitted the obtaining of clones of CHO cells producing HBsAg particles at a high level. This amplification has variable from one clone to another. In certain cases, the level of synthesis has been multiplied by 1500. For the clone concerned, the rate of production was 15 microgram/10<6 > cells per 24 hours. Several clones producing 1 to 10 micrograms of HBsAg/10<6 > cells per 24 hours were obtained. The rate of HBsAg production expressed per ml and per 24 hours depends on the culture conditions. The values of 10 to 20 micrograms/ml/24 hours could commonly be obtained, values comparable to the HBsAg concentration in the serum of chronic carriers. A kinetic study of the production of HBsAg has shown that this synthesis remains constant in stationary phase for several weeks. FIG. 7 reports the results of successive harvests gathered from cells in stationary phase, by periodic renewal of the culture medium over three weeks. Medium without serum may be used for these harvests. Other than the impact on cost price, the use of a medium without serum greatly facilitates the purification of the particles from the cellular supernatant and eliminates possible contaminations of seric origin. The synthesis of HBsAg by CHO clones has proved highly stable even in the absence of MTX. And this over a period of 6 months, around 300 generations. In certain cases, a slight spontaneous increase in HBsAg produced has even been observed. Never a diminution or a loss of the HBsAg production has been observed during gene amplification, and this on around 150 clones tested. These last two observations may be due to the structure of the vector in which the HBV sequences and the dhfr gene were only separated by

around 300 bp of pBR322.

The invention consequently permits the obtaining of clones which produce immunogenic particles of a particularly remarkable genetic stability.

A correlation between the number of copies of HBV sequences and the rate of HBsAg synthesis has not been observed. The technique utilized (the "dot blot") permits a global quantification, but does not permit discrimination of the functional sequences. The results obtained may be explained by the appearance of deletions and rearrangements in the vector during the transfection and amplification. This hypothesis is supported by the complexity of the electrophoretic profiles of the integrated HBV and dhfr sequences. Many of these sequences are probably non-functional. On the other hand there is perfect proportionality between the level of HBsAg synthesis and the quantity of specific HBV-RNAs. This demonstrates that the augmentation of the HBsAg synthesis observed is in fact due to an augmentation in the transcription of the HBV sequences, due in part very probably to an augmentation of the number of functional copies. This result also shows that the evaluation of the number of copies of an amplified gene, whether it be by dot blot or electrophoretic analysis is not the best test to evaluate the number of effective copies in a cellular clone. On the other hand, the quantification of the specific RNAs reflects perfectly the functional copies. This quantification takes on full interest in cases where the protein produced is not easily measurable.

The invention therefore concerns more particularly clones containing a dose of HBV-RNA of at least 2 x 10<3 > cpm/10<6 > cells in the system of measure such as has been described above for a probe having a specific activity of 10<5 > cpm/microgram (labeled with <32> P).

Two specific HBV-RNAs have been demonstrated. The initiation of the major transcript of 2.1 kb has been localized in the pre-S region at the position 3157. This is in agreement with the results of Catteneo et al (1983) Nature, vol. 305, 336-338, who have localized the initiation of the transcription of the S gene at this position. The minor RNA of 2.5 kb is initiated in the promoter of SV40. This RNA may be the mRNA of the polypeptide of 34,000 (GP34) present in the particles. It is therefore possible that the synthesis of GP34 in a large quantity (27% of the polypeptides of the particles) is due to the utilization of the early promoter of SV40 including the repeated 72 bp and placed 'upstream' of the pre-S region.

The utilization of a radioimmunoassay has shown that the particles synthesized by the CHO cells bearing the pHSA receptor. According to Machida et al (1983) Gastroenterology 85, 268-274, this receptor might be carried by the polypeptides GP31 and GP35. It is therefore probable that in our system the receptor is carried by GP34. The observed existence of only one polypeptide, and not two, carrier of the pHSA receptor in particles of cellular origin is not explained. It is possible that, contrary to seric particles, the glycosylation in the CHO cells is homogeneous.

The HBsAg particles of 22 nm synthesized by the CHO cells have the same immunogenicity as HEVAC B which is a preparation of particles of seric origin. The rates of HBsAg production obtained are compatible with an industrial utilisation. The presence of the pHSA receptor at the surface of these particles is a supplementary argument for the utilization of this system for the manufacture of a vaccine against hepatitis B.

As such, the invention concerns any vaccine composition against viral hepatitis B containing an effective dose of particles according to the invention, notably from 3 to 6 micrograms of protein/ml (unitary dose), in association with a pharmaceutical vehicle appropriate to the chosen mode of administration, notably parenterally.

CLAIMS: I claim:

[*1] 1. A composition useful as a vaccine, comprising substantially spherical particles, most of said particles comprise polypeptides having both immunogenic and immunologic characteristics of the HBsAg and a receptor for polymerized human albumin, and wherein said particles have sizes of 18 to 25 nm and densities permitting their isolation in a zone of 1.20-1.22 g/ml in a CsCl-based density gradient, and further wherein said composition:

(a) is free of Dane particles, HBe antigen, and HBc antigen;

(b) is essentially free of sera contaminants of human origin other than human albumin; and

(c) comprises said polypeptides at a proportion greater than 10% of the total quantity of the polypeptides comprising said particles.

[*2] 2. A composition useful as a vaccine, comprising substantially spherical particles, most of said particles comprise polypeptides having both immunogenic and immunologic characteristics of the HBsAg and a receptor for polymerized human albumin, and wherein said particles have sizes of 18 to 25 nm and densities permitting their isolation in a zone of 1.20-1.22 g/ml in a CsCl-based density gradient, and further wherein said composition:

(a) is free of Dane particles, HBc antigen, and HBe antigen;

(b) is essentially free of sera contaminants of human origin other than human albumin; and

(c) comprises said polypeptides at a proportion greater than 20% of the total quantity of the polypeptides comprising said particles.

[*3] 3. The composition according to claim 1, wherein said particles comprise polypeptides with molecular weights of about 34,000 daltons in a proportion greater than about 10% of the total quantity of the polypeptides comprising said particles.

[*4] 4. The composition according to claim 2, wherein said particles comprise polypeptides with molecular weights of about 34,000 daltons in a proportion greater than about 20% of the total quantity of the polypeptides comprising said particles.

[*5] 5. The composition according to claim 1, wherein said particles have sizes of from about 20 to about 22 nm.

[*6] 6. The composition according to claim 1, wherein said composition is free of components of human origin.

[*7] 7. The composition as claimed in claim 2, wherein said composition is free of components of human origin.

[*8] 8. A composition useful as a vaccine, comprising substantially spherical particles, most of said particles comprise polypeptides having both immunogenic and immunologic characteristics of the HBsAg and a receptor for polymerized human albumin, and wherein said particles have sizes of 18 to 25 nm and densities permitting their isolation in a zone of 1.20-1.22 g/ml in a CsCl-based density gradient, and further wherein said composition is obtained from a host cell transformed with an expression vector comprising an HBV DNA sequence comprising the S and pre-S regions of the genome of hepatitis B operatively linked to an exogenous promoter and said HBV DNA sequence does not comprise a gene encoding full length HBV core antigen.

[*9] 9. The composition as claimed in claim 8, wherein said host cell is the CHO cell.

[*10] 10. The composition as claimed in claim 9, wherein said exogenous promoter is the early promoter of SV40.

[*11] 11. The composition as claimed in claim 8, wherein said HBV DNA sequence is the 2.3 kb BglII restriction fragment of HBV DNA.

[*12] 12. A vaccine comprising an effective amount of a composition as in any one of claims 1 to 11, in association with a pharmaceutically acceptable vehicle.